Increased *WSB1* Copy Number Correlates with its Over-Expression Which Associates with Increased Survival in Neuroblastoma

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Gain of chromosome 17 is the most prevalent genetic abnormality identified in neuroblastoma (NB) and distal 17q gain has prognostic significance in NB. In this report, we have combined array-based comparative genomic hybridization (A-CGH) and gene expression analysis to investigate gene copy number changes and its impact on the gene expression level as well as their association with prognosis genes located on chromosome 17 in NB tumors. We observed differential gains of chromosome 17 between Stages 4- and 4S tumors. We found that *WSB1,* mapping to 17q11.1, which was frequently gained in 4S- tumors and not changed in 4- tumors, showed strong correlations between expression level and copy number. Furthermore, the increase of *WSB1* gene expression is associated with good outcome in patients with NB of all stages. *WSB1* also enhances the prognostic prediction when combined with other current prognostic factors in NB. Our results demonstrate that *WSB1* copy number correlates with its expression level and that its high expression associates with good prognosis suggesting a possible role of this gene in the biology of favorable outcome NB. This article contains Supplementary Material available at http://www.interscience.wiley.com/jpages/1045-2257/suppmat. Published 2006 Wiley-Liss, Inc.†

INTRODUCTION

Neuroblastoma (NB), the most common solid extra-cranial tumor of childhood, is characterized by diverse clinical behaviors ranging from spontaneous regression to rapid malignant progression (Brodeur, 2003). Many important factors, such as age, stage, *MYCN* amplification, and ploidy, have been shown to be associated with the biological and clinical heterogeneity of NB tumors. Although both Stage 4S and Stage 4 are metastatic tumors, the outcome of the diseases is radically different. Patients with Stage 4 tumors, in particular those with amplified *MYCN*, remain largely incurable despite advances in cancer therapeutics (Schwab et al., 2003), whereas the Stage 4S disease frequently regresses spontaneously and generally has a favorable outcome with little or no treatment (Brodeur, 2003). Stage 4S tumors occur only in infants under 1 year of age and are characterized by a unique metastatic pattern with dissemination primarily to liver and skin, and a small localized primary tumor. These diverse biological behaviors are often associated with particular genetic changes in NB (Chen et al., 2004, 2005; Bilke et al., 2005a). Many studies have reported a pronounced genetic heterogeneity of NB, and gain of chromosome 17 and 17q are the most frequent chromosomal abnormalities in NB (Chen et al., 2004; Bilke et al., 2005a; Vandesompele et al., 2005). Whole chromosome 17 gain is usually associated with the favorable prognostic factors, such as 3n ploidy, normal status of *MYCN* and 1p, and Stages 1, 2, and 4S. Conversely, gains of 17q21.1∼qter are found mostly in the 2n/4n ploidy tumors and are correlated with unfavorable prognosis (Trakhtenbrot et al., 2002; Vandesompele et al., 2005). In this study, we have combined array-based comparative genomic hybridization (A-CGH) and gene expression analysis to investigate gene copy number changes and expression level, as well as their association with prognosis, for genes located on chromosome 17 in NB tumors.

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MATERIALS AND METHODS

Tumors, Cell Lines, and Genomic DNA

Thirty-seven primary NB tumors used for CGH profiling were obtained from 15 patients in Stage 4S *MYCN* single-copy (4S-) and 22 in Stage 4 *MYCN* single-copy (4-). For the survival analysis, we used data from 49 primary NB tumor samples from a previously published study (Wei et al., 2004). Data from these tumors and additional samples from a second study (Wei et al., 2004; Krasnoselsky et al., 2005) were combined (total 112 samples) for the investigation of the association of the expression level *WSB1* with stage and *MYCN* amplification. This included 22 Stage 1 (1-), 17 Stage 2 (2-), 16 Stage 3 (3-), 9 Stage 4S (4S-), 31 Stage 4-, and 17 Stage 4 with *MYCN* amplification (4+). The original histological diagnoses were made at tertiary hospitals with extensive experience in diagnosis and management of neuroblastoma. The method for extracting RNA from tumors was done as described (Wei et al., 2004) and high molecular-weight genomic DNA was extracted as described (Chen et al., 2004).

Microarray Experiments

Preparation of glass cDNA microarrays, image acquisition, gene expression, and CGH experiment on cDNA microarray was performed as described (Chen et al., 2004; Wei et al., 2004). Images were acquired by an Agilent DNA microarray scanner (Agilent, Palo Alto, California), and analyzed using the Microarray Suite program, coded in IPLab (Scanalytics, Fairfax, Virginia). Microarray data analysis was performed as described previously (Chen et al., 2004; Wei et al., 2004). A total of 42,578 cDNA clones, representing 25,933 Unigene clusters, were on the cDNA microarray. After quality-filtering, we had 17,741 unique UniGene clusters remaining with 924 unique UniGene clusters on chromosome 17. For the A-CGH analysis, the genes were ordered according to their human genomic sequence position, and a moving average with a window of five on the CGH ratio was calculated. A multidimensional scaling method (MDS) was used to visualize the differences between the two groups in terms of the position of the samples in three-dimensional Pearson correlation space. MDS was performed using MATLAB.

Statistical Analysis for Evaluation of Prognosis

The probability of survival was calculated using the Kaplan–Meier method, and the significance of the difference between Kaplan–Meier curves was calculated using the Mantel-Haenszel method. Receiver operating characteristic (ROC) curve analysis was used to determine the cutoff value, based on optimum sensitivity and specificity in predicting poor outcome, to dichotomize the *WSB1* gene for subsequent evaluation. The gene expression level of *WSB1* used for ROC curve analysis was mean-centered log 2 ratio across all samples. The Cox proportional hazards model was used as described (Wei et al., 2004).

RESULTS

Differential Chromosome 17 Gains in 4S- and 4- Tumors

We performed A-CGH on 15 Stage 4S *MYCN*-not-amplified (4S-) and 22 Stage 4 *MYCN*-not-amplified (4-) NB tumor samples using cDNA microarrays. We used the copy number ratios of all 924 genes on chromosome 17 on our array to perform an MDS analysis. As shown in Figure 1A, 4S- and 4- tumors demonstrated distinct patterns of genomic alterations on chromosome 17. This was also shown in the probability heatmap (Fig. 1B). As reported previously (Vandesompele et al., 2005), most of 4S- tumors have either whole chromosome 17 gain or no gain. In the 4- group, we also observed two patterns of gain: whole chromosome gain or limited 17q gain, with most of 4- tumors showing 17q gain from 17q21.1(~34Mb)-17qter or 17q21.31(~41Mb)-17qter (Lastowska et al., 2002).

Increased *WSB1* Copy Number Correlated with High Gene Expression

We and others have observed the dramatic differential gains on chromosome 17 in 4S- and 4- tumors. To assess the impact of copy number gain of a gene on its mRNA expression level, we used the same microarray platform (Hyman et al., 2002; Pollack et al., 2002) to perform cDNA gene expression experiments on a subset of the tumors (22 NB tumors; 6 from 4S- and 16 from 4-) for which the mRNA was available. Pearson correlation analysis across the genes on chromosome 17 showed that 6.4% genes had correlation coefficient greater than 0.5 between copy number change and gene expression and that *WSB1*, located on 17q11.2, had the largest correlation coefficient between copy number change and gene expression [0.79, $P < 0.005$; Fig. 2A; see Supplemental Table 1 (Supplementary material for this article can be found at http://www.interscience.wiley.com/jpages/1045-2257/suppmat) for the correlation

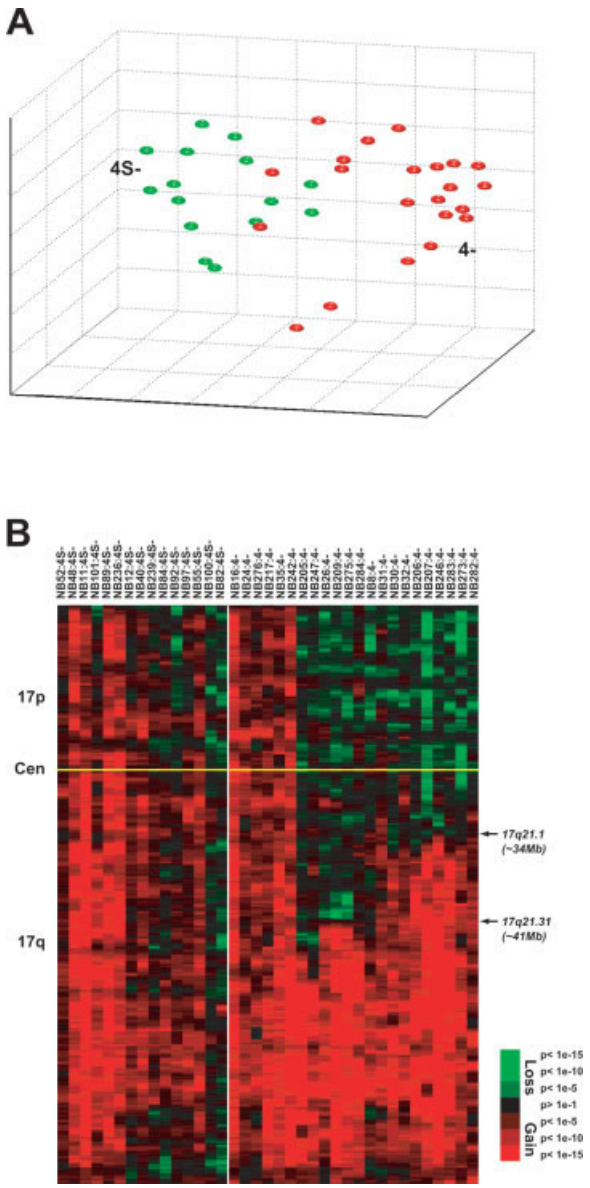

Figure 1. cDNA A-CGH analysis on chromosome 17 in Stages 4S- and 4- NB tumors. A. A three-dimensional plot produced by MDS displaying the similarity of DNA copy number in chromosome 17 among 37 tumor samples. All 924 genes that mapped to chromosome 17 were ordered according to genomic sequence position, and moving average with a window of five was performed on the pin-normalized A-CGH ratios prior to MDS analysis. Fifteen Stage 4S- tumors are shown in green and 22 Stage 4- tumors in red. B. Visualization of *P* values derived from the topological statistics as described in the previous papers (Chen et al., 2004; Bilke et al., 2005b) along the chromosome 17. Each column represents a different experiment, and each row represents the *P* value for the alteration at a given window of 20 adjacent clones ordered by genome map position from 17pter to 17qter. Red represents gain and green loss. The intensity of the color shows the level of significance according to the *P* value shown in the color scale. The arrows point to the positions of two possible breakpoints of 17q gain.

coefficients of top 200 genes on chromosome 17]. The expression of *WSB1* was validated by using Affymetrix HG-U133A array and a real time RT-PCR experiment [Supplementary Fig. 1 (Supplementary material for this article can be found at http://www.interscience.wiley.com/jpages/1045-2257/suppmat)].

Association of *WSB1* Expression with Prognosis

We next investigated the association of *WSB1* gene expression level with prognosis by using our previously published data set with known outcome information (Wei, et al., 2004) for the prognosis study. As shown in Figure 2B, *WSB1* was over-expressed in the samples with good prognosis compared with that in the samples with poor prognosis ($P < 0.0001$; Bonferroni corrected) indicating an association of *WSB1* with prognosis. We calculated the sensitivity and specificity of *WSB1* to predict outcome by using the ROC analysis (Dybowski et al., 1996). The ROC area for *WSB1* is 0.91 (Fig. 2C) indicating the high outcome predictive power of this gene. We also calculated the ROC area for all genes on chromosome 17; *PMP22* located on 17p12–p11.2 had the highest ROC area (0.95) followed by an EST (0.94) and *WSB1* (0.91; Fig. 2B) (see Supplementary Table 1 for the ROC areas). We combined ROC area and correlation coefficient information and calculated their product to identify the genes, which had both high correlations with gene copy number and high outcome predictive ability on chromosome 17. *WSB1* was the top gene with the product value of 0.73 ($P < 0.05$ assuming a Gaussian distribution for all positively correlated genes). Figure 2D shows the results for the top 15 genes (See Supplementary Table 1).

We next asked if the expression level of *WSB1* was associated with stage and *MYCN* amplification in a larger sample set of 112 tumors. As shown in Figure 3A, *WSB1* was over-expressed in Stages 1-, 2-, 3-, and 4S- as compared with that in 4- and 4+ tumors, indicating that the *WSB1* gene is strongly associated with stage and *MYCN* amplification.

Univariate proportional hazard analysis of the samples with survival data demonstrated that the hazard ratio (HR) of low expression (<0.2) of *WSB1* for death risk was 24.1 (95% CI: 3.20–180.0, $P < 0.0001$), which was higher than age (HR: 12.3, 95% CI: 1.6–92.5, $P = 0.0017$), stage (HR: 7.1, 95% CI: 2.1–24.2, $P = 0.0003$), and *MYCN* amplification status (HR: 9.8, 95% CI: 3.6–26.7, $P < 0.0001$), and similar to the currently used Children's Oncology Group (COG) clinical risk stratification status in North America (COG; HR: 29.7, 95% CI: 4.0–222.9, $P < 0.0001$) (Brodeur, 2002; Wei et al., 2004). Pair-wise comparison analysis demonstrated that *WSB1* expression level was significantly associated with all of these prognostic parameters (Table 1).

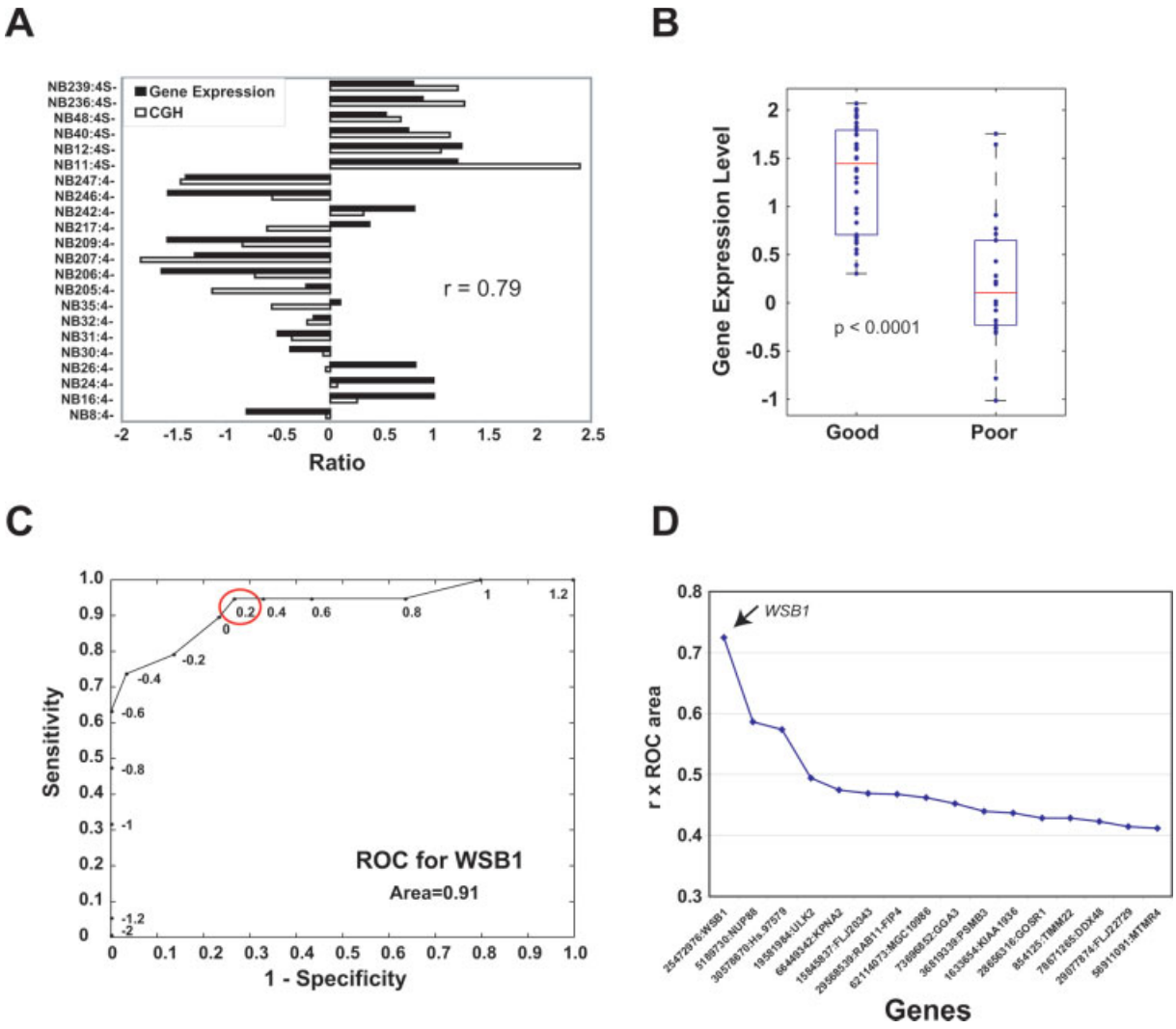

Figure 2. A. Correlation of DNA copy number with gene expression in chromosome 17. DNA copy number and gene expression ratio of *WSB1* in 4S- and 4- tumors are shown. Both A-CGH and gene expression ratio were log 2 scaled and z-scored. B. Differential expression of *WSB1* in good and poor prognosis patients. Box and whisker plots of the *WSB1* gene (log 2 ratio) are shown. Boxes represent the upper and lower quartile of the data. The red horizontal line within the box denotes the median. The whiskers extending above and below the box are fixed at 1.5 times the interquartile range. Outliers that fall outside the whiskers of the box are plotted as circles. C. ROC curve for *WSB1* was used to determine the cutoff value for optimum sensitivity and specificity to predict the outcome of samples in two different survival groups from a previously published dataset (Wei et al., 2004). The ratios shown under the curve are mean centered and log 2 scaled expression levels. The area under the curve was 0.91. D. Product of the gene copy number-expression correlation values and the ROC area for the top 15 genes. *X*-axis presents the genes (genome position on chromosome 17: gene symbol or use UniGene Cluster ID if no gene symbol is available). The arrow points to the top gene, *WSB1*. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

We also performed a Cox proportional hazards regression analysis including *MYCN* amplification, stage, and age. The Cox model showed that only *MYCN* amplification is significant in predicting survival in this cohort (Table 2A). When using either backward or stepwise selection, both *MYCN* and *WSB1* emerged as important factors that independently contributed to survival ($P < 0.05$, Table 2B). Additionally, a likelihood ratio test indicated a highly significant gain ($P2 = 0.00071$) when *WSB1* level was added to a model with *MYCN* amplification alone, which is a previously established important prognostic marker. Thus, *WSB1* may have potential value as a prognostic factor in addition to *MYCN* amplification.

Finally, we dichotomized the 49 NB samples based on their *WSB1* expression (threshold of 0.2 (mean-centered log 2 ratio across all samples), corresponding to a sensitivity of 95% and a specificity of 74% as determined from the ROC curve analysis (Fig. 2B)) and constructed a Kaplan–Meier survival curve (Fig. 3B). The results showed that the

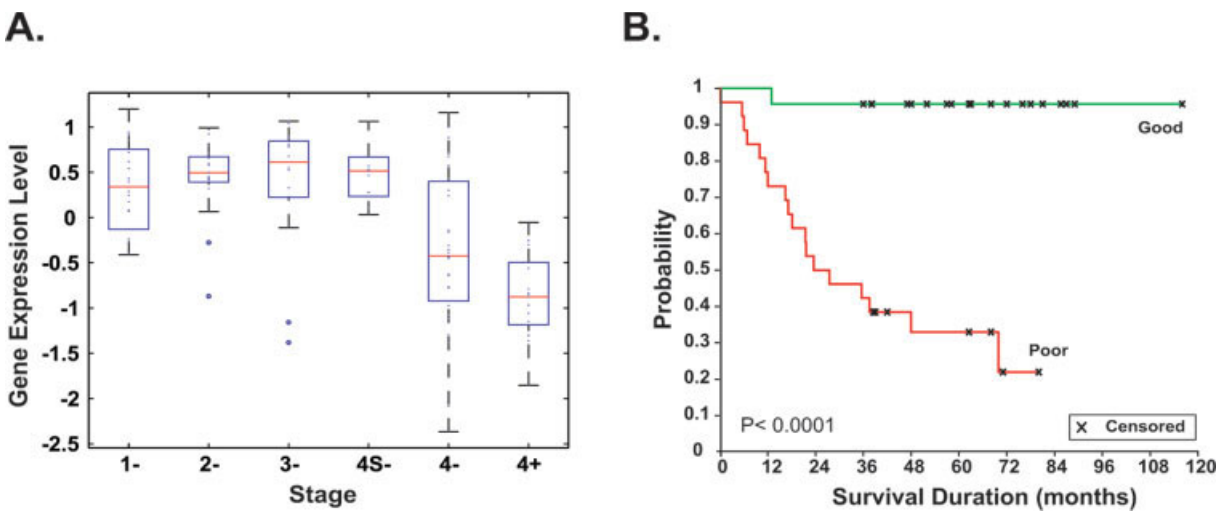

Figure 3. *WSB1* expression level is associated with stage and survival. A. Differential expression of *WSB1* in the different stages for 112 NB tumors. Box and whisker plots of the mean centered and log 2 scaled expression level of the *WSB1* gene are represented in the same way as Figure 2B. B. Kaplan–Meier curve of survival probability of 49 patients based on *WSB1* expression (<0.2 and >0.2). The patients classified by *WSB1* expression had significantly different survival probabilities based on the expression of *WSB1* ($P < 0.0001$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE 1. Association Between *WSB1* and Known Risk Factors

Parameters	*WSB1* expression*** High (> 0.2)	*WSB1* expression*** Low (< 0.2)	*P*-value (2-tailed)
Age			
<1.0	13	4	0.0025*
>1.0	10	22	
Stage			
1	9	2	<0.0001**
2	6	1	
3	4	2	
4	4	21	
MYCN			
Amplified	0	11	0.0004*
Not-amplified	23	15	
COG			
Low	15	3	<0.0001**
Intermediate	5	2	
High	3	21	

Abbreviation: COG, Children's Oncology Group risk stratification.
*Chi-Squared test.
**Cochran-Armitage test.
***Mean-centered and log 2 scaled expression level; threshold of 0.2 corresponds to sensitivity of 95% and specificity of 74% as determined from the ROC curve analysis (Fig. 2B).

patients with high and low expression levels of *WSB1* had significantly different survival probabilities ($P < 0.0001$, Fig. 3B).

DISCUSSION

We combined gene expression and DNA copy number data to select genes, which are likely to play an important role in the biology of NB. We focused on a known region of *recurrent* genomic alterations (chromosome 17) in NB, then subselected regions with differential probability of copy number alterations in two stages (4S- and 4-) and finally selected genes with a strong gene dosage effect. We have identified *WSB1* (17q11.1) as a top ranking candidate of such genes. Interestingly, we found that this gene is an independent marker significantly associated with survival in the cohort of patients used in this study. Remarkably this was found to be valid for a cohort of patients in *all* stages of NB and was not limited in the two stages (4S- and 4-), which were used to identify *WSB1*. *WSB1* also appeared in a gene list where the genes were differentially expressed between Stages 1 and 4 NB tumors in a previous study (Berwanger et al., 2002). These findings suggest an important role of *WSB1* in NB biology, which should be further evaluated in both laboratory and clinical setting. *WSB1* is a SOSC-box-containing WD-40 protein of unknown function; it is a Hedgehog-inducible ubiquitin ligase subunit and is capable of assembling with the Cul5/Rbx1 module to reconstitute potential ubiquitin ligases, which are involved in the protein degradation (Vasiliauskas et al., 1999; Dentice et al., 2005). *WSB1* is the primary E3 ubiquitin ligase for the thyroid-hormone-activating type II iodothyronine deiodinase (Dentice et al., 2005). It is possible that Hedgehog-induced *WSB1* may play an important role in the NB development through the regulation of protein degradation, but it needs further characterization.

TABLE 2. Multivariate Cox Proportional Hazard Model Analysis

Variable	Parameter estimate	Wald P2	Hazard ratio	95% CI for hazard ratio
A.				
Age (>1 vs. <1 year)	1.82	0.08	6.15	(0.85, 3.85)
Stage (4 vs. 1–3)	0.98	0.37	2.65	(0.32, 22.57)
MYCN (A vs. NA)	1.33	0.016	3.78	(1.27, 11.11)
WSB1 (Low vs. High)	2.01	0.066	7.45	(0.88, 63.30)
B.				
MYCN (A vs. NA)	1.37	0.0088	3.93	(1.41, 10.96)
WSB1 (Low vs. High)	2.71	0.011	15.01	(1.87, 120.07)

CI, confidence interval; A, amplified; NA, not amplified.

Gain of chromosome 17 is the most prevalent genetic abnormality identified in NB. The prognostic significance of distal 17q gain has been previously reported (Bown, 2001; Lastowska et al., 2002; Vandesompele et al., 2005). Whole chromosome 17 gain has been reported to be associated with favorable prognosis. Given that whole chromosome 17 gain and partial 17q gain have such a divergent association with prognostic effects, it is postulated that the important event is the imbalance between two or several genes on either sides of the breakpoint, rather than simple gain of a single gene (Bown, 2001). Currently, genes implicated in apoptosis, cell cycle control, and neuronal differentiation is of particular interest. *PPM1D,* which maps to 17q23, was reported to be a potential target for 17q gain in NB (Saito-Ohara et al., 2003). Survivin (*BIRC5*), which maps to 17q25, is a member of the inhibitor of apoptosis proteins and is significantly associated with poor prognostic factors (age and stage) and promotes cell survival in human NB (Islam et al., 2000). *NME1* and *NME2* located on 17q23.2 have also been reported as prognostic factors (Schramm et al., 2005). In contrast to the genes in the distal 17q region for which their high expression is associated with poor prognosis, we have herein shown that high expression of *WSB1* in the proximal 17q region (17q11) is associated with good prognosis. These results suggest that the balance of genes localized on proximal 17q (e.g., 17q11–17pter) and distal 17q (e.g., 17q21-qter) may play an important role in controlling the chromosome 17 gain effect.

In summary, we have combined A-CGH and gene expression analysis to investigate gene copy number changes and expression level as well as their association with prognosis for all genes located on chromosome 17 on our array. We identified that *WSB1*, mapping to 17q11.1, had a gene dosage effect and that its high expression is associated with good prognosis. Such a gene dosage alteration may play an important role in controlling the chromosome 17 gain effect in NB. Furthermore, we showed that *WSB1* holds promise to enhance the prognosis prediction when combined with the current prognostic factors in NB.

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